Comparative transcriptome combined with morpho-physiological analyses revealed key factors for differential cadmium accumulation in two contrasting sweet sorghum genotypes

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Summary
Cadmium (Cd) is a widespread soil contaminant threatening human health. As an ideal energy plant, sweet sorghum (Sorghum bicolor (L.) Moench) has great potential in phytoremediation of Cd-polluted soils, although the molecular mechanisms are largely unknown. In this study, key factors responsible for differential Cd accumulation between two contrasting sweet sorghum genotypes (high-Cd accumulation one H18, and low-Cd accumulation one L69) were investigated. H18 exhibited a much higher ability of Cd uptake and translocation than L69. Furthermore, Cd uptake through symplasmic pathway and Cd concentrations in xylem sap were both higher in H18 than those in L69. Root anatomy observation found the endodermal apoplasmic barriers were much stronger in L69, which may restrict the Cd loading into xylem. The molecular mechanisms underlying these morpho-physiological traits were further dissected by comparative transcriptome analysis. Many genes involved in cell wall modification and heavy metal transport were found to be Cd-responsive DEGs and/or DEGs between these two genotypes. KEGG pathway analysis found phenylpropanoid biosynthesis pathway was over-represented, indicating this pathway may play important roles in differential Cd accumulation between two genotypes. Based on these results, a schematic representation of main processes involved in differential Cd uptake and translocation in H18 and L69 is proposed, which suggests that higher Cd accumulation in H18 depends on a multilevel coordination of efficient Cd uptake and transport, including efficient root uptake and xylem loading, less root cell wall binding, and weaker endodermal apoplasmic barriers.

Introduction
As global industrialization proceeds, cadmium (Cd) has become one of the most harmful and widespread pollutants in environment (He et al., 2013; Xue et al., 2014). Cadmium is easily taken up by plants and accumulates in edible parts, thus posing risks to humans health through food chains (Uruguchi and Fujiwara, 2013). Various strategies have been developed to remove Cd from polluted soils, among which phytoremediation is regarded as an environmentally friendly, cost-effective and in situ remediation technology (Krämer, 2005). Although hyperaccumulators such as Thlaspi caerulescens are effective in extracting metals from soil (Deng et al., 2007; McGrath et al., 2006; Singer et al., 2007; Song et al., 2012), most hyperaccumulators have small biomass, slow growth rate and low economic benefit, which greatly restrict the development of phytoremediation technology (Douchiche et al., 2012; He et al., 2013). Nowadays, it is suggested that some fast growing, metal tolerant and high biomass plant species, especially energy plants, may be more effective in phytoextraction of heavy metals from contaminated soils (Juwarkar et al., 2008). Sweet sorghum (Sorghum bicolor (L.) Moench), a C₄ plant with high photosynthetic efficiency (Gnansounou et al., 2005; Yu et al., 2012), is resistant to stresses such as salt, drought, heat and toxic pollution. Sweet sorghum is widely cultivated in tropical, subtropical and temperate regions (Marchiol et al., 2007; Zhuang et al., 2009). In addition, it has high biomass yield and high sugar content in its stalk, making it an ideal feedstock for ethanol production (Bennett and Anex, 2009; Calvino and Messing, 2012; Gnansounou et al., 2005). Phytoremediation with sweet sorghum can not only combine soil remediation with bioenergy production, but also transfer heavy metals from the food chain into the energy chain, thus avoiding the harm to human beings (Jia et al., 2016; Li, 2013; Woods, 2001). Previous studies have
shown that sorghum was more tolerant to Cd and copper (Cu) treatment than wheat and maize (Metwali et al., 2013). What’s more, sorghum can accumulate large quantities of Cd, Cu, lead (Pb) and zinc (Zn) in shoots as its biomass was higher than that of sunflower or corn (Zhuang et al., 2009). However, as nonhyperaccumulator, most of the absorbed Cd was retained in the roots of sweet sorghum plants, while only a small amount can be transported to the shoots (Angelova et al., 2011; Jia et al., 2016; Soudek et al., 2014). To improve the capacity of phytoremediation, it is of great importance to promote the capacity of Cd accumulation in its shoots.

Several physiological processes determine the Cd accumulation in the shoots of plants, including root uptake, sequestration into vacuoles and translocation in the xylem as well as phloem. Cadmium in soil enters plants via apoplastic and symplasmic pathways (Lu et al., 2009). It is generally accepted that active acquisition of Cd occurs mainly via uptake systems for essential elements, such as Fe$^{2+}$, Ca$^{2+}$, Zn$^{2+}$ and Mn$^{2+}$ (Lombi et al., 2002; Pence et al., 2000; Perfus-Barbeoch et al., 2002). However, a specific mechanism of Cd uptake mediated by high-affinity Cd transporters was also suggested in T. caerulescens (Zhao et al., 2002). After absorption by the roots, Cd is transported to the stele by passing through endodermis (Lux et al., 2011) and then translocated to shoot via xylem, the latter of which is driven by transpiration from leaves (Lu et al., 2009; Lux et al., 2011). Further accumulation of Cd into seeds or grains is mediated by phloem transport as illustrated by several studies (Kato et al., 2010; Uraguchi et al., 2011). Previous studies on the response of sweet sorghum to Cd stress were mostly descriptive and limited to the physiological level. Currently, the uptake, translocation and accumulation processes of Cd in this valuable energy plant are still mostly unknown. In this study, using two contrasting sweet sorghum genotypes, the mechanisms responsible for their differential Cd accumulation were investigated through a combination of physiological, microstructural and comparative transcriptome analyses.

**Results**

Sweet sorghum genotypes H18 and L69 exhibited a great difference in the capacity of Cd uptake and translocation.

In previous study, we identified two sweet sorghum genotypes with contrasting Cd translocation factor (TF) from ninety-six germplasm obtained from USDA (United States Department of Agriculture), that is UMM EL TEIMAN (Accession NO. PI 152873, designated as H18), with highest TF and MN 4578 (Accession NO. PI 273969, designated as L69) with lowest TF (in submission). To compare the effects of cadmium on H18 and L69 plants, the growth parameters were first detected. Under both control and Cd conditions, the biomass of L69 was slightly higher than that of H18 (Figure S1a). Cd treatment suppressed the growth of H18 and L69 seedlings. Although the plant height reduction was comparable between H18 and L69 (Figure S1b), the dry weight reduced about 40% in L69, which was much more than that in H18 (about 15%) (Figure S1c). In addition, the photosynthetic rate and chlorophyll contents decreased after Cd treatment, both with lower values in L69 than in H18 (Figure S1d,e). Furthermore, most of the absorbed Cd was retained in the roots of H18 and L69 seedlings, yet the Cd content of root and shoot was both higher in H18 (Figure 1a). Moreover, the Cd translocation factor of H18 was about fourfold as high as L69 (Figure 1b). These results indicate that H18 has much higher Cd accumulation and translocation capacity than L69. Metals can be absorbed by plant roots both passively and actively, and thus, the effect of low temperature (4 °C) and metabolic inhibitor CCCP on Cd accumulation was investigated as symplasmic pathway was predicted to be minimal under these conditions (Zhao et al., 2002). Low temperature or addition of CCCP both significantly inhibited Cd uptake in H18 and L69 roots, yet the inhibitory effect was much more pronounced in H18 (Figure 1c,d). The Cd accumulation was comparable in H18 and L69 roots under 4 °C (Figure 1c) while it was significantly lower in H18 than L69 after CCCP treatment (Figure 1d). As net symplasmic uptake can be estimated by subtracting the apparent uptake at 4 °C or under CCCP treatment from that of 25 °C, these results indicated higher symplasmic Cd uptake by root in H18 than in L69.

Scanning ion-selective electrode technique (SIET) was also used to investigate Cd$^{2+}$ influx. We scanned 0–1200 μM from root tips of H18 and L69 and detected the highest Cd influx rate at 300 μM (Figure 2a). Thus, this site was selected for Cd$^{2+}$ influx detection in the subsequent analysis. Marked differences were found between the two genotypes while the Cd$^{2+}$ influx value in H18 roots was 2.5-fold of that in L69, indicating higher Cd uptake capacity in H18 roots compared to L69 (Figure 2b,c).

**Endodermal apoplastic barriers were much stronger in L69 than those in H18.**

After absorption by the roots, Cd is transported to the stele by passing through endodermis (Lux et al., 2011). We thus observed the root anatomy structure through semi-thin and ultrathin section microscopy. Cd treatment caused considerable anatomical alterations in H18 roots (Figure 3a,c), whereas the anatomy structure of L69 changed a little (Figure 3b,d). Under Cd condition, the endodermis became falcate in H18 roots, and the xylem was more concentrated surrounding only one pole compared to three poles in control (Figure 3a,c). However, the endodermis kept normal plump shape in L69 roots and xylem differentiation was slightly affected (Figure 3d). Through transmission electron microscopy, we observed intensive U-shaped thickenings of endodermal cells in Cd-treated roots of H18 and L69 (Figure 3i–p). However, U-shaped thickenings were found in all endodermal cells of L69 roots (Figure 3m–p). In contrast, in H18 roots, some endodermal cells without U-shaped thickenings (Figure 3i,j) or with thinner cell walls (Figure 3k,l) were found. When quantified, the endodermal U-shaped thickenings in L69 roots were significantly thicker (1.03 ± 0.14) than those in H18 roots (0.57 ± 0.09 and 0.29 ± 0.06) (Figure 3q). These results indicated that endodermal apoplastic barriers were much stronger in L69 than those in H18 under Cd stress.

**Cd contents in xylem sap was much higher in H18 than that in L69.**

To examine the translocation ability of Cd into the shoot, xylem sap was collected from H18 and L69 plants. The collected xylem sap had a total protein concentration of 10–14 μg/mL (Table S1), being comparable with maize xylem sap (12 μg/mL) (Alvarez et al., 2006) but far lower than phloem sap values (200 μg/mL in rice and Lupinus species to 35–60 mg/mL in Curcubitaceae) (Rodriguez-Celma et al., 2016). In addition, it contained very low concentrations of sucrose, glucose and fructose (Table S1), which was characteristic of xylem saps but was different from the phloem extrudes containing high concentrations of sucrose (238 mM in castor bean) (Hall and Baker, 1972; Smith and
Milburn, 1980; Ye et al., 2010). Both of these results showed good purity of the collected xylem sap with very low possibility of contamination from phloem sap.

Then, Cd concentrations in collected xylem sap were determined. The exposure to Cd decreased xylem sap transport in both genotypes although the decline was not significant, and the volume of xylem sap in H18 and L69, which was detected at 300 μm from the root tip for 10 min. (c) The mean Cd⁺⁺ influxes in root cells within 10-min measuring periods. Values are means ± SE (n = 3). Asterisk indicates significantly different from those of H18 (Student’s t-tests, P < 0.05). Cd interferes with the accumulation of micronutrients in plants. Thus, the effects of Cd on Fe, Zn, manganese (Mn), Cu, calcium (Ca) and magnesium (Mg) accumulation in H18 and L69 plants were investigated. The changes of Fe and Cu contents had the same tendency, which increased significantly in roots while not changed in shoots, and the values in roots were higher in H18 than in L69 (Figure S2a,d). The accumulation of Zn and Mn did not change in roots while decreased in shoots of both H18 and L69 (Figure S2b,c). The contents of Ca increased in both roots and shoots of H18, but remained unchanged in L69 (Figure S2e). In addition, Mg accumulation decreased in the roots of both H18 and L69 while increased only in H18 shoots (Figure S2f).

The accumulations of Fe, Zn, Mn, Cu, Ca and Mg in xylem exudate were also detected, among which Zn and Cu contents were not affected by Cd (Figure S3b,d) while Fe and Mn contents decreased (Figure S3a) and increased (Figure S3c), respectively, only in L69. In addition, after Cd treatment, Ca content in xylem increased in H18 yet decreased in L69 (Figure S3e), while Mg was only up-regulated in H18 (Figure S3f).

Key factors for Cd accumulation in sweet sorghum

Figure 1 Cd uptake and accumulation was much higher in H18 than in L69. (a, b) H18 and L69 seedlings were treated with 0 and 10 μM CdCl₂ for 2 weeks, and Cd content in shoot (S) and root (R) was detected (a), and translocation factor was calculated by (Cd content in shoot)/(Cd content in root) (b). (c, d) H18 and L69 seedlings were transferred to uptake solution with or without 10 μM CdCl₂ at 25 and 4 °C (c) or 100 μM CCCP (d). After 24 h, Cd contents in roots were detected. Values are means ± SE (n = 3 and five plants for each replicate). Different letters above the bars indicate significant differences at P < 0.05. For (b), double asterisks above the column indicate significant difference (Student’s t-tests, P < 0.01).
Comparative transcriptome analysis identified key genes and processes responsible for differential Cd accumulation between H18 and L69

To further elucidate the molecular basis for the differential Cd accumulation in H18 and L69, we conducted comparative transcriptome analysis through high-throughput digital gene expression (DGE) sequencing. A 24-h treatment of 10 μM Cd exposure was used to investigate early response of sweet sorghum to Cd stress. Twelve DGE libraries were created from three biological replicates each for H18 and L69 roots under control and CdCl₂ treatments and sequenced. After removing low-quality reads and those containing adapter and poly-N, more than 10 million clean reads remained in each sample (Table S2),
among which more than 90% was mapped to the *Sorghum bicolor* genome (http://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Sbicolor_er) (Table S3).

Then, differentially expressed genes (DEGs) were identified through comparisons of the FPKM values for each gene between H18 and L69 (L69CK/H18CK and L69Cd/H18Cd) or between Cd-treated and CK samples in each genotype (H18Cd/H18CK and L69Cd/L69CK), and thus, DEGs between two genotypes and DEGs involved in Cd response were screened, respectively. Under normal conditions, 1095 genes expressed differentially between H18 and L69, while this value reached 1743 after Cd treatment, among which 873 genes were common ones, indicating genetic differences between the two genotypes (Figure 5a,b, Table S4).

For Cd-responsive DEGs, a total of 389 genes were differentially expressed in H18 after Cd treatment, including 344 up-regulated and 45 down-regulated genes. However, 1962 Cd-responsive DEGs were found in L69, including 1553 up-regulated and 409 down-regulated ones (Figure 5c). Among them, 313 genes were common Cd-responsive genes (Figure 5d).

To evaluate the validity of deep-sequencing data, five Cd-responsive genes were selected for expression levels examination by qRT-PCR (Table S11). The results were consistent with that of deep sequencing, with a positive correlation ($R^2 = 0.6989$, $P = 0.003$) indicating the reliability of high-throughput data (Figure S4).

**Gene ontology (GO) analysis of DEGs**

To identify the major functional categories represented by the DEGs, GO enrichment analysis was carried out using the singular enrichment analysis (SEA) in agriGO program (Du et al., 2010). For DEGs between H18 and L69, GOs associated with oxidation reduction in the biological process category (Figure 6a) and the ones associated with heme binding, iron ion binding, oxidoreductase activity, tetrapyrrole binding, electron carrier activity as well as ADP binding in the molecular function category (Figure 6b) were significantly enriched under both control and Cd stress conditions. For Cd-responsive DEGs, GO items of oxidation reduction in the biological process category (Figure 6c), and heme binding, tetrapyrrole binding, peroxidase activity, oxidoreductase activity as well as iron ion binding in the molecular function category (Figure 6d) were enriched in both H18 and L69.

**KEGG pathway enrichment of DEGs**

To further systematically understand the molecular interactions among the DEGs, we performed KEGG analysis (Kanehisa and Goto, 2000). For DEGs between H18 and L69, pathways of phenylpropanoid biosynthesis, carbon fixation in photosynthetic organisms, photosynthesis and ribosome were enriched under both control and Cd conditions (Figure 7a, Table S6). Cd-responsive DEGs in both H18 and L69 were enriched in phenylpropanoid biosynthesis and biosynthesis of amino acids pathways. Besides, the protein processing in endoplasmic reticulum, glutathione metabolism, phenylalanine, tyrosine and tryptophan biosynthesis, pentose phosphate pathway, as well as biosynthesis of secondary metabolites were specifically enriched for Cd-responsive DEGs in H18. In contrast, plant hormone signal transduction, DNA replication, cell cycle, cysteine and methionine metabolism, metabolic pathways, pantothenate and CoA biosynthesis, alpha-linolenic acid metabolism, cutin, suberin and wax biosynthesis, starch and sucrose metabolism as well as flavonoid biosynthesis were uniquely enriched for Cd-responsive DEGs in L69 (Figure 7b, Table S6).

**Genes involved in phenylpropanoid and lignin biosynthesis**

KEGG analysis showed phenylpropanoid biosynthesis was over-represented both for Cd-responsive DEGs and DEGs between two
genotypes (Figure 7), indicating that this pathway may play important roles in Cd response of sweet sorghum and the differential Cd accumulation between H18 and L69. We thus focused on the 38 DEGs involved in this pathway, among which 13 were Cd-responsive DEGs, 15 were DEGs between H18 and L69, while the remaining 10 were not only Cd-responsive but also differentially expressed between the two genotypes (Figure 8a, Table S7).

Other genes involved in cell wall (CW) metabolism

CW is the primary structure directly exposed to Cd, especially in roots. The transcriptome data identified other 57 DEGs involved in CW metabolism and modification (Figure 8b) besides the aforementioned 38 DEGs involved in lignin biosynthesis (Figure 8a), including seven involved in CW biogenesis, 10 involved in CW modification, eight involved in CW organization, nine involved in CW macromolecule catabolic process, two involved in pectin metabolic process, 14 involved in cellulose biosynthesis and catabolism, as well as six involved in suberin biosynthesis (Figure 8b). Among these 57 DEGs, 32 were Cd-responsive DEGs and 18 were DEGs between H18 and L69. For the 32 Cd-responsive DEGs, 22 were up-regulated by Cd and 10 were down-regulated, and most (26 of 32) were specifically responsive to Cd in L69 roots. The remaining seven genes were Cd-responsive DEGs, which also expressed differentially between two genotypes. Interestingly, six of them were expressed higher in L69 (Table S8).

Genes involved in heavy metal transport

As transporters play important roles in Cd uptake, transport and sequestration process, 59 DEGs encoding heavy metal transporters were also examined, which can be divided into seven groups (I–VII) based on their expression patterns (Figure 9, Table S9). The first group consisted of eight genes whose expressions were significantly up-regulated in both H18 and L69 roots under Cd treatment (Figure 9a). The three genes in the second group and the eighteen genes in the third group were specifically up-regulated in H18 and L69 roots by Cd treatment, respectively (Figure 9b,c). Genes in the fourth group were down-regulated by Cd treatment either in both genotypes or specifically in L69 (Figure 9d). The expression of genes in the fifth group was higher in L69 roots than those in H18 (Figure 9e). The sixth group had eleven genes expressed higher in H18 than those in L69 (Figure 9f). Groups I to IV were Cd-responsive DEGs whereas groups V and VI were DEGs between two genotypes. The seven genes in Group VII were Cd-responsive DEGs between H18 and L69. Three genes separately encoding copper chaperone homolog (CCH), copper transporter (COPT) and ABCA transporter were up-regulated by Cd in both H18 and L69. Moreover, their expressions were higher in L69 roots than those in H18 (Figure 9e). The sixth group had eleven genes expressed higher in H18 than those in L69 (Figure 9f). Groups I to IV were Cd-responsive DEGs whereas groups V and VI were DEGs between two genotypes. The seven genes in Group VII were Cd-responsive DEGs between H18 and L69. Three genes separately encoding copper chaperone homolog (CCH), copper transporter (COPT) and ABCA transporter were up-regulated by Cd in both H18 and L69. Moreover, their expressions were higher in L69 than in H18. Two major facilitator superfamily genes were up-regulated by Cd specifically and expressed higher in L69 roots by Cd treatment. Genes encoding a yellow stripe-like protein (YSL) and an OPT transporter were up-regulated by Cd only in H18 roots, and YSL expressed higher in L69 while OPT expressed higher in H18, respectively (Figure 9g).

Discussion

As an ideal feedstock for ethanol production, sweet sorghum has great potential in phytoremediation of Cd-polluted soils (Jia et al., 2016; Li, 2013; Woods, 2001). However, progress in improving phytoremediation ability of this species is limited as the regulatory
mechanisms of Cd uptake, accumulation and transport in sweet sorghum remain largely unexplored. Two sweet sorghum genotypes, H18 and L69, with contrasting Cd translocation factors were used in this study, with H18 collected from Sudan while L69 from Ethiopia. Although significant genetic differences were found as more than 1000 DEGs were identified between them (Figure 5), through comparing Cd-responsive genes in H18 and L69 and identifying Cd-responsive DEGs that expressed differentially between them, key factors for differential Cd accumulation were identified, which were consistent with the morpho-physiological results.

Transporters are involved in differential root uptake and xylem translocation capacity of Cd between H18 and L69.

Based on the physiological data (Figures 1, 2 and 4), we reasoned that differential root uptake and xylem loading capacity of Cd may be main factors determining the contrasting Cd accumulation in H18 and L69. Uptake of Cd from the external solution to root cells and following translocation via xylem is thought to be mediated through transporters, but transporters for Cd have not been identified in sweet sorghum. Several studies have reported that transporters for essential elements such as Fe$^{2+}$, Zn$^{2+}$ and Ca$^{2+}$ may be involved in Cd uptake and transport (Lux et al., 2011). In total, we identified 59 transporter-encoding DEGs, among which 35 were Cd-responsive DEGs and seven were Cd-responsive DEGs which were also expressed differentially between H18 and L69 (Figure 9, Table S9).

Cd can enter root cells in the form of Cd$^{2+}$ through ZIP (Zinc-regulated transporter/iron-regulated transporter-like protein) transporters, which are capable of transporting a variety of divalent cations, including Cd$^{2+}$, Fe$^{2+}$, Zn$^{2+}$ and Mn$^{2+}$ (Guerinot, 2000; Plaza et al., 2007). In this study, four ZIP encoding genes were found to be DEGs, among which Sb01g013660 was induced by Cd in both genotypes, Sb01g012440 was induced by Cd only in H18, while Sb02g006960 and Sb10g022390 were specifically induced by Cd in L69 (Figure 10). This result indicates that uptake of Cd in H18 and L69 roots may involve different ZIP transporters.

Nramp (natural resistance-associated macrophage protein) family members function as general metal ion transporters which

Figure 7 KEGG analysis of DEGs between H18 and L69 under CK and CdCl$_2$ conditions (a) and DEGs following CdCl$_2$ exposure (b). The x-axis indicates the percentage of DEGs numbers vs. background gene numbers in each KEGG pathway. The detailed information is shown in Table S6.
can transport Fe\(^{2+}\), Zn\(^{2+}\), Mn\(^{2+}\), Cu\(^{2+}\), Ni\(^{2+}\), Co\(^{2+}\) and Cd\(^{2+}\) (Nevo and Nelson, 2006). For example, OsNramp5 functions as a major transporter responsible for root Mn and Cd uptake in rice (Sasaki et al., 2012). A very recent study reported that HvNramp5 is a transporter for Mn and Cd uptake in barley, which is localized in plasma membrane and constitutively expressed in the root tip epidermal cells (Wu et al., 2016). In this study, we found about fourfold and 1.5-fold up-regulated expression of \(Sb\text{Nramp5 (}Sb02g007870\) by Cd treatment in H18 and L69, respectively; however, it was not DEGs as judged by our criteria. This result is similar to HvNramp5, whose expression is not affected by Cd despite it shows transport ability for Cd\(^{2+}\) (Wu et al., 2016). In addition, report on OsNramp1 showed that it might assist xylem loading of Cd for root to shoot mobilization (Tiwari et al., 2014). A \(Sb\text{Nramp gene (}Sb02g007890\) homologous to \(Os\text{Nramp1 was up-regulated under Cd stress in both H18 and L69 roots, indicating that it might be involved in Cd translocation in sweet sorghum. However, no difference in expression was found for \(Sb02g007890\) between the two genotypes. Besides, higher expression of another \(Sb\text{Nramp gene (}Sb01g043400\) homologous to \(At\text{Nramp2/3/4/6 was detected in L69 both constitutively and under Cd stress. \(At\text{Nramp3 and \(At\text{Nramp4} were localized in} \) vacuolar membrane and function in export of metals (Fe, Zn, and Mn) from the vacuolar compartment to the cytosol (Lanquart et al., 2004, 2005, 2010; Thomine et al., 2003). \(At\text{Nramp6 functions as an intracellular metal transporter, whose presence, when modified, is likely to affect the distribution of Cd within the cell. \(At\text{Nramp6 over-expressed Arabidopsis plants were hypersensitive to Cd, although Cd content remained unchanged (Cailliatte et al., 2009). We suggest that \(Sb01g043400\) may play important roles in Cd tolerance through export of Cd from vacuolar to cytosol in sweet sorghum. However, the exact role needs to be further investigated.

In addition to Cd\(^{2+}\), Cd can enter root cells as Cd-chelates through YSL (yellow stripe 1-like) proteins (Curie et al., 2009), which are also involved in the loading of Cd from symplasm into xylem (Verbruggen et al., 2009). In this study, three YSL-encoding genes (\(Sb04g033320, Sb04g033330\) and \(Sb04g033340\) were identified to be DEGs (Figure 10). Interestingly, the former two were induced by Cd only in roots of H18, the high-Cd accumulation genotype, and the expression of \(Sb04g033320\) was up-regulated for even more than 40-fold. We inferred that these genes may play essential roles in Cd uptake and/or xylem loading in genotype H18. Further study of the exact molecular function may help reveal the mechanism of differential Cd accumulation in the two sweet sorghum genotypes.

\(P_{\text{ip}}\)-type ATPase (heavy metal transporting ATPase, HMA) proteins also play important roles in xylem loading of Cd. Arabidopsis has eight members of HMA family proteins, among which \(At\text{HMA3}\) is involved in sequestration of Zn and Cd into vacuoles (Williams and Mills, 2005), while \(At\text{HMA2 and \(At\text{HMA4 are implicated in xylem loading of Cd and Zn (Hanikenne et al., 2008; Mills et al., 2003; Papoyan and Kochian, 2004; Verret et al., 2004). Through BLASTP analysis, we identified 10 candidate genes encoding HMA in \(S. bicolor\) genome (Table S10). However, only one of them, \(Sb04g006600\), was found to be induced by Cd treatment in both genotypes and its expression was comparable between H18 and L69. This result indicated that although \(Sb04g006600\) may play roles in Cd response in sweet sorghum, its involvement in differential Cd accumulation in H18 and L69 may not be based on the regulation of expression but on the different functions, just as the recent report on Os\(HMA3.\) Yan et al. found a loss-of-function allele of \(Os\text{HMA3 with a predicted amino acid mutation from Ser to Arg at the 380th position, which was associated with high-Cd accumulation in shoots and grain of a} \text{Japonica rice cultivar (Yan et al., 2016).}

Enhanced cell wall (CW) binding and endodermal apoplastic barriers in L69 may restrict Cd translocation to shoot

CW is the first barrier to toxic metals in the environment, whose synthesis and composition are affected by heavy metals.

**Figure 8** Heat map of DEGs involved in phenylpropanoid pathway and lignin biosynthesis (a) as well as cell wall modification (b). Relative expression was calculated by Log2Ratio. H18\(\text{CK, H18Cd, L69\text{CK and L69Cd represent the H18 and L69 roots treated with 0 and 10 \mu g CdCl}_2, respectively. The detailed information is shown in Tables S7 (a) and S8 (b).**
Here, we found the apoplastic Cd uptake was significantly lower in H18 than in L69 (Figure 1d). Although Cd concentration in CW was a little lower in L69, the CW yield in L69 was much higher under Cd treatment (Figure S5), resulting in higher total Cd content in L69 root CWs. As CW is the major pool of heavy metal accumulation in roots (Fernández et al., 2014), a higher binding capacity of CW to Cd in L69 will result in a lower distribution of Cd to the shoot. After uptake by roots, the transport of Cd to shoot is controlled by apoplastic barriers in endodermis (Schreiber, 2010), whose differentiation generally includes three stages: the formation of Casparian strips, suberin lamellae and U-shaped tertiary walls (Meyer et al., 2009; Redjala et al., 2011). Exposure to Cd can induce acclamatory responses of endodermis, including extensive thickening of the inner tangential walls of endodermal cells (Degenhardt and Gimmler, 2000), formation of Casparian strips and suberin lamellae closer to the root apex (Martinka and Lux, 2004; Schreiber et al., 1999; Vaculík et al., 2009; Zelko and Lux, 2004), increase of suberin and lignin contents as well as the alteration of their chemical composition (Schreiber et al., 1999). All these changes can reduce apoplastic movement of Cd to xylem and its translocation to shoot. In this study, U-shaped tertiary walls were found in endodermis of both H18 and L69 roots under Cd stress (Figure 3i–p), indicating that Cd treatment could induce and enhance the suberization and lignification of the endodermis. It is worth noting that the endodermal apoplastic barriers was much stronger in L69 roots, as the tertiary walls of endodermis were significantly thicker in L69 (Figure 3q) and there were no visible passage cells in L69 (Figure 3m–p) in contrast to many passage cells in H18 roots (Figure 3i,j). Passage cells are endodermal cells with unsuberized tangential walls, usually found opposite the xylem poles. Although the exact functions are uncertain, available evidence suggests that passage cells are important for transfer of Ca and Mg into stele and thus into the transpiration stream (Peterson and Enstone, 1996). We proposed that the stronger endodermal apoplastic barriers in L69 can prevent a higher proportion of Cd through apoplastic transport into xylem. In

Figure 9 Heat map of DEGs encoding heavy metal transporters. Relative expression was calculated by Log2Ratio. H18_CK, H18_Cd, L69_CK and L69_Cd represent the H18 and L69 roots treated with 0 and 10 μM CdCl₂, respectively. The detailed information is shown in Table S9.
contrast, passage cells in H18 endodermis may render the transport of Cd into stele more efficiently.

In accordance with the morpho-physiological results, many DEGs identified by transcriptome data were found to be linked with CW modification, including genes involved in CW biogenesis and modification as well as CW macromolecule (pectin, cellulose, lignin and suberin) catabolic process. In addition, KEGG analysis showed over-representation of phenylpropanoid biosynthesis pathway, the product of which serves as metabolite source for lignin biosynthesis (Vogt, 2010). Interestingly, most of these DEGs were specifically Cd-responsive DEGs in L69 or expressed higher in L69, including 21 of 38 DEGs involved in symplasmic pathway and genes involved in Caspian strip formation were shown. Numbers 1, 2, 3 and 4 represent relative expression calculated by Log2(H18Cd/H18CK), Log2(L69Cd/L69CK), Log2(L69CK/H18CK) and Log2(L69Cd/H18Cd), respectively. Grey box represents the expression of the gene was not changed significantly.

Based on the above results and previous studies, a schematic representation of main processes involved in differential Cd uptake and translocation in H18 and L69 plants was proposed (Figure 10). Our results suggest that higher Cd accumulation in sweet sorghum genotype H18 than in L69 depends on a multilevel coordination of efficient Cd uptake and transport, which is determined by efficient root uptake and xylem loading, less root cell wall binding, and weaker endodermis apoplastic barriers. This study can not only lay the foundation for further understanding of varied CW components might be different between H18 and L69 roots, resulting in different binding capacity of Cd to CW. On the other hand, discriminating ultrastructure of apoplastic barriers in endodermis, imposed by differences in biological processes such as lignin and suberin biosynthesis and formation of Caspian strip, may also contribute to differential Cd uptake and translocation.
exploring the molecular mechanisms of Cd accumulation in plants, but also provide new strategies for improving phytoremediation ability of energy plants through genetic engineering.

**Experimental procedures**

**Plant materials and growth conditions**

Sweet sorghum genotypes H18 and L69 were obtained from Plant Genetic Resources Conservation Unit, USDA, Griffin (http://www.ars-grin.gov/npgs/index.html). Sweet sorghum plants were grown hydroponically according to the conditions used by Jia et al. (2016).

**Low temperature and metabolic inhibitor treatment**

Two-week-old hydroponically grown H18 and L69 seedlings were transferred to uptake solution containing 0.5 mM CaCl₂ and 2 mM MES (pH 5.8) with or without 10 μM CdCl₂ for different treatments including: control (25 °C), low temperature (4 °C) and Cd + 100 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP). Before the uptake experiment at 4 °C started, plants were pretreated with an ice-cold uptake solution for 30 min and uptake containers were placed in an ice bath. After 24 h, plants were rinsed with uptake solution, which were used to determine Cd concentrations.

**Determination of Cd and other micronutrients contents**

The roots of H18 and L69 seedlings were washed with distilled water to remove surface ions, and then, roots and shoots were harvested separately, which were dried at 70 °C until constant weight, and were grounded to fine powder and digested in 2 mL nitric acid at 180 °C for 4 h. The concentrations of Cd and mineral elements were determined using ICP-AES (Thermo Fisher Scientific, 81 Wyman Street, Waltham, MA, USA).

**Root anatomical structure and ultrastructure observation**

Ultrathin sections of roots were obtained as described by Jia et al. (2016) and observed under a JEM-1230 electron microscope (JEOL, Tokyo, Japan). The semi-thin sections (1 mm) were cut using an ultramicrotome (Leica Microsystems, Wetzlar, Germany) and were stained with 1% (w/v) toluidine blue in 1% (w/v) borax. Images were taken using a Zeiss Axiosplan II compound microscope with a Maximal Ratio Combiner digital camera and AxioVision software (Carl Zeiss, Jena, Germany).

**Measurement of Cd²⁺ flux**

The net Cd²⁺ flux was measured using SIET (BIO-001A; Younger USA Sci. & Tech. Corp., Beijing, China) as described by Xu et al. (2012).

**Collection of xylem sap and detection of Cd and mineral elements contents**

Xylem sap was collected as described previously (Deng et al., 2013; Lu et al., 2013; Ueno et al., 2008; Wu et al., 2015). H18 and L69 seedlings were treated by 10 μM CdCl₂ for 24 h, which were de-topped at 2 cm above the shoot-root junction. The surface of the excised leaf sheath was gently wiped, and after discarding the initial 1–2 μL of exudates, a tube filled with cotton was placed on the cut end and wrapped with Parafilm. The xylem sap was collected for 8 h, which was then weighed and stored at −20 °C until further analysis. Twenty-four plants were used for xylem sap collection, and six individual samples each were pooled at random into four tubes. Forty microliter xylem samples were diluted to 10 mL, and the contents of Cd and mineral element were analysed by ICP-MS or ICP-AES.

**Digital gene expression (DGE) tag sequencing**

**cDNA library construction and sequencing**

Two-week-old H18 and L69 seedlings were treated with 0 (CK) and 10 μM CdCl₂ (Cd) for 24 h. Then, roots of eight seedlings were collected for RNA extraction. Each treatment had three biological replicates, resulting in a total of 12 samples. Total RNA was extracted with RNAiso™ plus (TaKaRa Bio Inc., Otsu, Shiga, Japan) and treated with RNase-free DNase I (Fermentas, Thermo Scientific, Waltham, MA) to remove the residual DNA. The quantity of RNA was checked using the NanoDrop ND-1000 (Thermo Scientific, Wilmington, DE) and 1.2% agarose gels. The integrity of the total RNA was assessed using Agilent 2200 Tape Station (Agilent Technologies, Santa Clara, CA), and each sample had an RNA integrity number (RIN) > 8.0.

cDNA libraries were constructed using the Illumina Truseq RNA sample preparation Kit (Illumina Inc., San Diego, CA) following the manufacturer’s recommendations, which were then sequenced by the Illumina HiSeq™ 2500 platform (RiboBio Co. Ltd, Guangzhou, China).

**Sequencing data analysis—Quality control**

The raw data were first filtered through in-house Perl scripts to obtain high-quality clean reads. Simultaneously, the Q20, Q30 and GC content levels of the clean data were evaluated. The data sets generated and/or analysed during the current study are available in the NCBI SRA repository under accession number SRP080196 (http://www.ncbi.nlm.nih.gov/sra/SRP080196).

**Reads mapping and gene annotation**

The reference genome and gene model annotation files were directly downloaded from NCBI. The FM index of the reference genome was built using Bowtie (Broad Institute, Cambridge, MA). The clean reads were then aligned to the reference genome using TopHat (Broad Institute). During the alignment of reads, seven mismatches and a gap length of 7 bp were allowed. The functional annotations of genetic variants were generated using ANNOVAR.

**Quantification of gene expression level**

Gene expression levels were measured via RNA-seq analysis as a unit of expected number of fragments per kilobase of transcript sequence per million base pairs sequenced (FPKM) (Trapnell et al., 2010). DESeq software was used to identify DEGs (Anders and Huber, 2010). The DESeq analysis was performed by fitting normalized count data to a generalized linear model (GLM) estimating a negative binomial distribution of the calculated mean values of the three biologically independent samples. The sequences with fold change > 2 and P_adj < 0.05 (P values adjusted for multiple testing with the Benjamini-Hochberg procedure) were deemed to be DEGs.

**GO and KEGG pathway analyses**

GO enrichment analysis was performed by the agriGo program (http://bioinfo.cau.edu.cn/agriGO). The GO terms in SDEGs with corrected P < 0.05 were defined as significantly enriched. KEGG pathway analysis was carried out using the KOBAS software (KOBAS, Surrey, UK).
Statistical analysis

One-way ANOVA were performed using the SPSS 17.0 program. Differences were tested by Duncan’s test unless otherwise specified.

Acknowledgements

The study was supported by The National Key Research and Development Program of China (2017YFD0801105) and The Science and Technology Program for Public Wellbeing (2013GS460202-X). We thank Fengqin Dong (Key Laboratory of Plant Molecular Physiology, IBCAS) for technical assistance on transmission electron microscopy. The authors declare that they have no competing interests.

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Key factors for Cd accumulation in sweet sorghum


Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1 Effects of cadmium on the growth of H18 and L69 seedlings.

Figure S2 Effects of Cd treatment on micronutrients accumulation.

Figure S3 Effects of Cd treatment on micronutrients contents in xylem sap.

Figure S4 qRT-PCR validation of expression profiles of DEGs.

Figure S5 The effect of Cd treatment on cell wall yield (a) and Cd concentration in CW (b) in H18 and L69 roots.

Table S1 The total protein concentrations and sugar composition in xylem exudates of H18 and L69 plants.

Table S2 Sequencing output statistics.
Table S3 Mapping of RNA-seq reads to the reference *S. bicolor* genome sequence.
Table S4 List of differentially expressed genes (DEGs).
Table S5 Enriched GO categories of the DEGs.
Table S6 KEGG enrichment results of the DEGs.
Table S7 DEGs involved in phenylpropanoid and lignin biosynthesis.
Table S8 DEGs involved in cell wall metabolism.
Table S9 DEGs encoding possible heavy metal transporters.
Table S10 The *HMA* genes in *S. bicolor* genome and their expression profiles in H18 and L69 roots under control and 10 μM CdCl₂ conditions.
Table S11 The primers used in qRT-PCR analysis.
Appendix S1 Supplemental methods.